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X-ray Spectroscopic Observation of an Interstitial Carbide in NifEN-bound FeMoco Precursor

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Abstract

The iron-molybdenum cofactor (FeMoco) of nitrogenase contains a biologically unprecedented μ_6 -coordinated C^{4-} ion. Although the role of this interstitial atom in nitrogenase catalysis is unknown, progress has been made on understanding its biosynthetic origins. Here, we report valence-to-core (V2C) Fe K β X-ray emission spectroscopy (XES) to show that this C^{4-} ion is present in the Fe₈S₉ “L-cluster,” which is the immediate precursor to FeMoco prior to the insertion of molybdenum and coordination by homocitrate. These results accord with recent evidence supporting a role for the S-adenosylmethionine (SAM)-dependent enzyme NifB in the incorporation of carbon into the FeMoco center of nitrogenase.

The iron-molybdenum cofactor (FeMoco) of nitrogenase is Nature’s most active catalyst for the conversion of inert N₂ to bioavailable NH₄⁺.^{1,2} A complex metallo-cluster with a Fe₇S₉Mo core, the FeMoco is coordinated in the host protein by homocitrate and a histidine at the Mo end and a cysteine at the opposite Fe end (Figure 1b). It also contains an interstitial light atom that was identified recently as a carbide (C^{4-}) ion.^{3,4} The role of the interstitial C^{4-} is unclear and, so far, no synthetic models have emerged to assist in the elucidation of its contribution to nitrogenase activity. Unraveling when and how this atom is incorporated into the cofactor structure, therefore, is crucial for a better understanding of its function.

Biosynthesis of FeMoco starts with the formation of Fe₂S₂ and Fe₄S₄ fragments on NifS and NifU, followed by the formation of a precursor from these small FeS fragments on NifB. This precursor, or the “L-cluster” (Figure 1b), is transferred to NifEN.⁵ Maturation to the catalytically active FeMoco is then achieved by metal substitution of an apical Fe for Mo and coordination of homocitrate.

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ASSOCIATED CONTENT

Supporting Information

Methods for sample preparation and XES measurement and analysis, full K β XES spectra for all samples, and fits to difference spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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A recent report has shown that ^{14}C can be traced from the S-methyl moiety of SAM to NifB, then to NifEN, and ultimately to the active MoFe protein.⁶ Multiple lines of evidence indicate that the couriers of this radiolabel are the L-cluster and FeMoco, clearly demonstrating that the C^{4-} in FeMoco originates from SAM. However, incomplete structural characterization leaves the mode of association of the ^{14}C with the L-cluster unclear.

The structure of L-cluster was initially described as either a 7 or 8 Fe core on the basis of EXAFS studies. Subsequent EXAFS studies of the isolated L-cluster established an 8 Fe core and identified a light atom contribution that could be attributed to either the interstitial atom or solvent.⁷ Crystallographic analysis provided further support for an Fe_8S_9 model of this cluster, although the 2.6 Å resolution structure could also not unambiguously indicate the presence or absence of an interstitial light atom.⁸ Moreover, structural data at this resolution cannot dismiss the possibility that one of the μ^2 S-donors is methylated. We note that EXAFS and NRVs have been used to argue for the presence of an interstitial light atom in NifB-co, a proposed FeMoco precursor.^{9–11} However, NifB-co is likely irrelevant to FeMoco biosynthesis, particularly in light of recent results which have shown that the L-cluster can be directly assembled from Fe_4S_4 clusters without proceeding through NifB-co.¹² We thus chose to focus our studies on NifEN, which binds the immediate precursor to FeMoco, thus allowing us to temporally deduce the point of carbon insertion.

Valence-to-core (V2C) Fe K β X-ray emission spectroscopy (XES) is a powerful technique to probe the identity of inner-sphere ligands,^{3,13–18} and does not require crystalline samples or isotopic enrichment. V2C XES measures the spectrum of photons emitted when electrons populating ligand-centered molecular orbitals (MOs) are demoted energetically to fill a Fe 1s core hole, generated by X-ray induced photoionization. V2C XES features arising from transitions from MOs of predominantly ligand ns parentage are referred to as K β '' transitions and serve as elemental fingerprints because these MOs minimally participate in chemical bonding and, as such, are primarily sensitive to atomic ionization energies. Light atom K β '' bands are well-separated: for O, N, and C, occurring near 7092, 7096, and 7100 eV, respectively.^{3,15} Moreover, the requirement of mixing and, therefore, overlap with Fe 4p orbitals to confer intensity to these transitions means that only inner-sphere atoms contribute.^{16–17} Additionally, V2C XES can differentiate charged states of inner-sphere donors. For example, the N 2s V2C bands of N^{3-} , HN^{2-} , and H_2N^- are predicted to be shifted by 1 eV per protonation.¹⁸ The utility of this method renders it the most suitable approach to ascertain the presence of an interstitial atom in the NifEN-bound L-cluster.

Here, we investigate the V2C XES of NifEN (containing the Fe_8S_9 L-cluster and the Fe_4S_4 "O-cluster") and $\Delta nifB$ NifEN (containing only the O-cluster). The $\Delta nifB$ variants of *Azotobacter vinelandii* do not produce NifB, which is required to convert the Fe_4S_4 clusters to the Fe_8S_9 L-cluster on NifB. Consequently, FeMoco biosynthesis is stalled at a step prior to the sequential formation of L-cluster and FeMoco, resulting in the production of $\Delta nifB$ NifEN (L-cluster-deficient) and $\Delta nifB$ MoFe protein (FeMoco-deficient) that can be used for the analysis of background Fe-S contributions to V2C XES data. Our present results mark a more rigorous spectroscopic deconvolution of interstitial atom contributions to V2C XES than removal of an empirical S^{2-} contribution as we reported previously.³ However, the same net result is achieved.

We have directly subtracted V2C XES data of $\Delta nifB$ NifEN from those of extracted FeMoco, which permitted an unbiased correction for background X-ray fluorescence. Since $\Delta nifB$ NifEN binds only the Fe_4S_4 "O-cluster", subtraction of its spectrum has also allowed us to remove the S^{2-} contributions from the V2C data in correct proportion to the number of emissive Fe atoms. RS^- contributions to the spectra are expected to be insignificant relative

to S^{2-} , as we have previously demonstrated.³ Thus, any intensity arising from an interstitial species can be isolated in this manner. Indeed, this procedure reveals significant residual intensity in FeMoco at 7099.8 ± 0.1 eV (Figure 2a, gray) that is consistent with the previously reported value of the interstitial C^{4-} in FeMoco.³ Likewise, subtraction of the V2C data of $\Delta nifB$ NifEN from those of NifEN reveals residual intensity at 7099.7 ± 0.4 eV (Fig. 2b, red) that is indicative of the presence of interstitial C^{4-} in the precursor. Subtraction of NifEN from FeMoco removes all intensity from the 7090–7103 eV region (Figure 2b, blue), providing further confirmation for the presence of a C^{4-} in both clusters.

In order to better compare the FeMoco and the L-cluster in their native protein environments with the isolated FeMoco, we have also performed subtractions of V2C data of $\Delta nifB$ MoFe protein and $\Delta nifB$ NifEN from those of MoFe protein and NifEN, respectively, thus removing the corresponding P-cluster and O-cluster contributions to the spectra (Figure 3). While these spectra still possess the 2s feature of C^{4-} at 7100 eV, they highlight differences in the higher energy regions of the spectra. In particular, deconvolution of contributions to spectral intensity in the 7103–7115 eV region, which comprises V2C transitions from occupied MOs of ligand np parentage, is complicated by substantial metal-ligand MO mixing. Regardless, data derived from these subtractions indicate a perturbation of the environment of L-cluster relative to those of the native and extracted FeMoco. We propose that the greater intensity near 7105 eV exhibited by the NifEN-bound L-cluster could originate from the binding of H_2O to exposed Fe sites, resulting in an O 2p contribution to the XES spectrum at ~ 7105 eV.^{14,15} We also suggest that the differences between the spectra of the isolated FeMoco (in NMF) and the native FeMoco (within the MoFe protein environment) may reflect an impact of NMF-binding on the electronic structure of the cofactor. Specifically, replacement of cysteine by NMF in extracted FeMoco will replace some S 3p contributions to this region by N 2p contributions.

In summary, we have employed V2C XES to establish the presence of the interstitial C^{4-} in the Fe_8S_9 L-cluster, which is the immediate precursor to FeMoco prior to the insertion of molybdenum and homocitrate. Our results indicate that at this stage of the biosynthesis the carbon species is fully deprotonated and bound to the iron. The observation of a NifEN-associated Fe_8S_9C cluster at this stage of biosynthesis is consistent with its incorporation via a NifB-catalyzed, radical-SAM dependent mechanism.¹² The exact mechanism of such a process, as well as its adaptation to synthetic model chemistry, merits further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Core structures of the precursor (PDB entry: 3PDI) in NifEN (a) and the FeMoco (PDB entry: 3U7Q) in MoFe protein (b). The interstitial C^{4-} atom is included in both structures. (c) Structure of the O-cluster in NifEN. Atoms are colored as follows: S, yellow; Fe, orange; C, black; Mo, cyan.

Figure 2.

(a) V2C XES spectra of extracted FeMoco (grey), NifEN (red), and $\Delta nifB$ NifEN protein (blue). (b) Difference spectra between extracted FeMoco and $\Delta nifB$ NifEN (gray), NifEN and $\Delta nifB$ NifEN (red), and extracted FeMoco and NifEN (blue). Smoothed spectra are overlaid in dashed black lines for clarity.

Figure 3. V2C XES spectra of isolated FeMoco (black), MoFe protein corrected for P-cluster contributions (red), and NifEN protein corrected for O-cluster contributions (blue).